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Supercritical Fluid Chromatography

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Introduction

Great emphasis is currently placed on differences in biological activities, potencies and toxicities of enantiomeric pharmaceutical compounds. The US Food and Drug Administration (FDA) has recently implemented regulations for the enantiomeric purity of enantiomeric drugs and chemicals. This has led to the development of chromatographic methods for the enantiomeric resolution of racemates including gas chromatography, liquid chromatography, and more recently supercritical fluid chromatography (SFC).

The physicochemical properties of enantiomers are the same except when they are placed in an asymmetric environment. This can be obtained before the chromatographic column or within the column by using a chiral derivatizing agent in the mobile phase or by using a chiral stationary phase.

Formation of Diastereomers by Using a Pre-Column Derivatization

In this method, the racemate is reacted with an optically pure compound leading to formation of diastereomers. Owing to their different physiocochemical properties, diastereomers can be resolved by using classical achiral mobile and stationary phases. This method can only be applied to molecules bearing reactive functions such as amines, acids and alcohols. For preparative purposes, partial racemization can occur when recovering the initial enantiomer. This problem represents the major limitation of this method. Moreover, this method has some disadvantages: (1) the chiral reagent must be optically pure, or its optical purity has to be well known (otherwise, poor accuracy will be achieved); (2) the derivatizing reaction must be quick and quantitative; and (3) the chromatographic behaviour of the derived diastereomers should be suitable (easy separation, good stability under the chromatographic conditions, ease of recovery with absence of racemization during the step leading to the initial enantiomers).

This was the method of choice before the development of chiral stationary phases (CSPs). It is still applied, but usually in order to improve detection limits. The method is not commonly used with SFC although (*S*)-trolox methyl ether has been used to derivatize chiral alcohols for attempted separation by GC and SFC with achiral systems. Using this derivatizing method, several compounds were successfully resolved by SFC but GC failed because of thermal decomposition of the ester derivatives.

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Formation of Labile Diastereomers in the Mobile Phase

This method generally consumes chiral reagent. Moreover, the major limitation concerns detection, which must be compatible with the nature of the chiral reagent contained in the mobile phase. In the case of preparative applications the limitation is related to the recovery of the sample, which must be separated from the chiral reagent. Although, the optical purity of the reagent has no effect on the accuracy of the results, it decreases the selectivity of the method. One example of the use of SFC in this way is the chiral separation of amino alcohols using chiral ion pairing (**Figure 1**). In this case SFC analysis time was significantly less than that for high performance liquid chromatography (HPLC) separation.

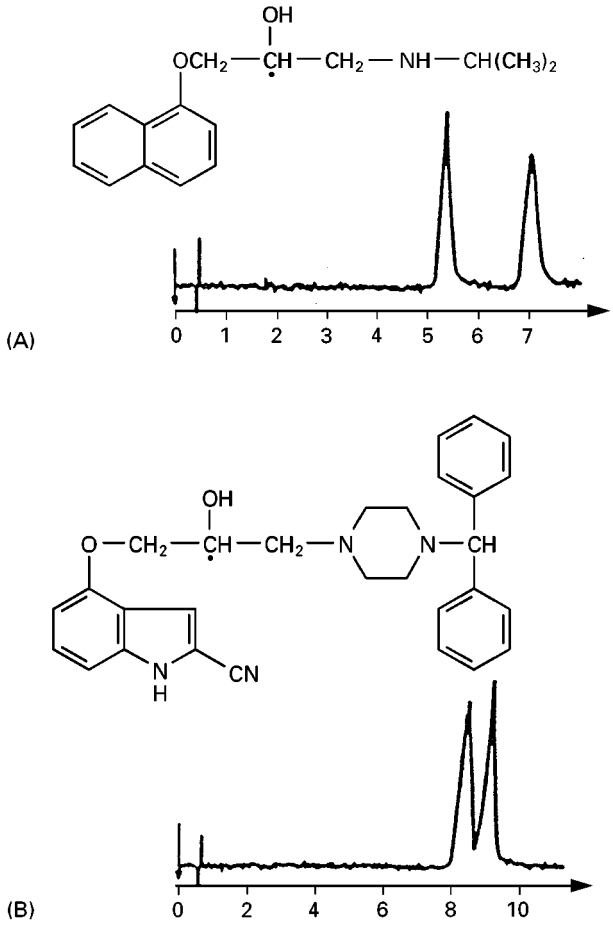

Figure 1 Chiral separation of propranolol (A) and DPI 101–106 (B) using ion pair SFC. Operating conditions: 100 × 4.6 mm i.d. column packed with 5 μm cyanopropyl-grafted silica (Brownlee GS–GU); mobile phase: carbon dioxide/acetonitrile (80 : 20, v/v) containing 5×10^{-3} mol L^{-1} of triethylamine and 3.5×10^{-2} mol L^{-1} of *N*-benzoxycarbonylglycyl-1-proline; pressure 250 bar; temperature: 21°C. (Reproduced from Steuer W, Schindler M, Schill G and Erni F (1988) Supercritical fluid chromatography with ion-pairing modifiers. Separation of enantiomeric 1,2-aminoalcohols as diastereomeric ion pairs. *Journal of Chromatography* 447: 287–296, with permission from Elsevier Science.)

Use of Chiral Stationary Phases

As in HPLC, SFC with chiral stationary phases, which was described for the first time by Mourier and colleagues, is the most powerful technique for the separation of enantiomers.

Capillary Columns

The coupling of SFC-CSPs can be performed either with capillary or packed columns. However, few applications have been described using capillary columns since the number of commercially available GC-CSPs is low and setting up the back-pressure regulator is somewhat difficult to adjust precisely with a capillary column. Packed columns provide greater scope for applications, mainly due to the greater number of chiral stationary phases commercially available and their ease of use. Consequently only SFC on chiral packed columns will be described here.

Packed Columns

CSPs designed for HPLC are widely used in SFC. This is because separations are performed at room temperature so that there is greater interaction energy between CSP-racemate (and therefore higher selectivities) with fewer racemization problems. Packed-column SFC has the same advantages and this is why, since 1985, this technique has been successfully applied to chiral separations. However, the lack of commercial equipment for packed-column SFC has long been a major problem in the development of the technique; this handicap is now being overcome. The advantages of SFC over HPLC include: faster analysis, faster column equilibration, faster method development and also reduced generation of hazardous waste. It must also be underlined that SFC sometime exhibits thermodynamic advantages over HPLC by providing greater selectivity values (particularly with natural polymer CSPs).

Chiral Stationary Phases

The first commercial LC-CSP was described in 1981 and more than 100 CSPs are now commercially available (**Table 1**). These CSPs can be divided into four groups, depending on their chemical structure and the chiral recognition mechanisms involved.

Group I Group I CSPs are divided into two subgroups. Brush-type CSPs (Pirkle-type and analogues, constitute the first subgroup (IA in **Table 1**). They are the most amenable to scientific investigation because they work as independent CSPs, since each chiral graft operates independently in distinguishing the solute enantiomers.

Ligand-exchange CSPs (subgroup IB in **Table 1**) cannot be used in SFC because the formation of the ternary complex, chiral selector–Cu^{II}–solute, takes place almost exclusively in an aqueous medium.

Group II This group contains cyclodextrin CSPs and crown ether CSPs. Only the cyclodextrins have been applied in SFC (**Table 1**).

Group III The chiral selector is here a polymer, natural (as with amylose and cellulose) or synthetic (as with polyacrylamide) bearing a lot of stereogenic centres and asymmetric cavities. The formation of the

Table 1 Commercially available CSPs

Chiral selector	*Commercial name*	*Supplier*
Type IA		
(*R*)- or (*S*)-(3,5-Dinitrobenzoyl)phenylglycine	DNBPG	B, R
	ChiralDNBPG-C	Ser
	Sumichiral OA-2000	Sum
	Sumichiral OA-2000S	Sum
(*R*)- or (*S*)-*N*-(3,5-Dinitrobenzoyl)tyrosine *n*-butylamide	ChyRoSine-A	Sed
(*S*)-(*S*)-*N*-(3,5-dinitrobenzoyl)tyrosine [1-(1-naphthyl)-ethyl]amide	ChyRoSine-AD	Sed
(*S*)-(3,5-Dinitrobenzoyl)leucine	DNBLeu	B, R
	ChiralDNBL-C	Ser
(*S*)-(3,5-Dinitro-benzoyl)phenylalanine	Chiraline	SFCC
(*R*)-α-Methylbenzyl urea	Supelcosil-LC-(*R*)-urea	Sup
(*R*)- or (*S*)-*N*-(2-Naphthyl)alanine		R
(*S*)-α-(1-Naphthyl)ethylamine	Sumichiral OA-1000	Sum
(*R*)-Phenylglycine amide derivative and (*S*)-(4-(4-Chlorophenyl) isovaleric acid derivative	Sumichiral OA-2100	Sum
(*R*)-Phenylglycine amide derivative		
(1*R*-3*R*)-Chrysanthemic acid derivative	Sumichiral OA-2200	Sum
(*R*)- or (*S*)-1-(3,5-Dinitrobenzoyl)naphthyl glycine	Sumichiral OA-2500	Sum
	Sumichiral OA-2500S	Sum
(*S*)-Valine t-butyl urea	Sumichiral OA-3000	Sum
(*S*)-(3,5-Dinitrobenzylurea)valine	Sumichiral OA-3100	Sum
(*S*)-(3,5-Dinitrobenzylurea)*tert*-leucine	Sumichiral OA-3200	Sum
(*S*)-Valine-(*S*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4000	Sum
(*S*)-Valine-(*R*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4100	Sum
(*R*)-Phenylglycine-(*R*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4200	Sum
(*R*)-Phenylglycine-(*S*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4300	Sum
(*S*)-Proline-(*S*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4400	Sum
(*S*)-Proline-(*R*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4500	Sum
(*S*)-*t*-Leucine-(*S*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4600	Sum
(*S*)-*t*-Leucine-(*R*)-[1-(1-naphthyl)ethyl]urea	Sumichiral OA-4700	Sum
Tartric acid and 3,5-dinitrobenzylphenylethylamine	Nucleosil Chiral-2	MN
Dimethyl *N*-3,5-dinitrobenzoyl-α-amino-2,2-dimethyl-4-pentyl phosphonate	(*R*)-α-Burke 1	B, R
(*S*,*S*)- or (*R*,*R*)-1-[(3,5-Dinitrobenzoyl)amino) 2-allyl-1,2,3,4-tetrahydrophenanthrene	(*S*,*S*) or (*R*,*R*) Whelk-O 1	B, R
N-3,5-Dinitrobenzoyl-3-amino-3 phenyl-2-(1,1-dimethylethyl)propanoate	β-GEM 1	B, R
Type IB		
Silica-grafted amino acids (proline, valine, hydroxyproline)	Chiral hydroxyCu	Ser
	Chiral proCu	Ser
	Chiral valCu	Ser
	Nucleosil Chiral-1	MN
	Chiralgel L-prolinamide	MN
	Chiralgel L-valinamide	MN
	Chiralgel L-phenylalinamide	MN
	Chiralpak WM/WE	D
	Chiralpak MA (+)	D
	Accusphere	JW
1,2-(2-Carboxymethylamino)-diphenyl ethanol	Chiralpak WE	D
Type IIA		
α-Cyclodextrin	Cyclobond III	A
β-Cyclodextrin	Cyclobond I	A
	Chiradex	M
	Chiral β-dex	Ser
γ-Cyclodextrin	Cyclobond II	A
Acetylated α-cyclodextrin	Cyclobond III Ac	A
Acetylated β-cyclodextrin	Cyclobond I Ac	A
β-Cyclodextrin derived (*S*)-2-hydroxy-propyl	Cyclobond I SP	A
β-Cyclodextrin derived 2-hydroxy-propyl (racemic)	Cyclobond I RSP	A
β-Cyclodextrin derived (*S*)-[1-(1-naphthyl)ethyl]carbamate	Cyclobond I SN	A
β-Cyclodextrin derived (*R*)-[1-(1-naphthyl)ethyl]carbamate	Cyclobond I RN	A
β-Cyclodextrin derived [1-(1-naphthyl)ethyl]carbamate (rac)	Cyclobond I RSN	A
β-Cyclodextrin derived 3,5-dimethylphenylcarbamate	Cyclobond I DMP	A
β-Cyclodextrin derived 4-methylphenylcarbamate	Cyclobond I PT	A

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Table 1 *Continued*

Chiral selector	*Commercial name*	*Supplier*
Type IIB		
Grafted silica crown ether	Crownpak CR(+)	D
Type IIIA		
Triacetylated microcrystalline cellulose (raw polymer)	Cellulose triacetate	M
	Chiral triacel	MN
	Chiralcel CA-1	D
Cellulose triacetate	Chiralcel OA	D
Tribenzoate cellulose	Chiralcel OB; OB-H	D
Triphenylcarbamate cellulose	Chiralcel OC	D
Tri(3,5-dimethylphenyl)carbamate cellulose	Chiralcel OD; OD-H	D
	Chiralcel OD-R (reversed-phase)	D
Type IIIA		
Tri(4-chlorophenyl)carbamate cellulose	Chiralcel OF	D
Tri(4-methylphenyl)carbamate cellulose	Chiralcel OG	D
Tri(4-methylbenzoate)cellulose	Chiralcel OJ	D
Tricinnamate cellulose	Chiralcel OK	D
Tri(3,5-dimethylphenyl)carbamate amylose	Chiralpak AD	D
Tri-(*R*)-(1-phenylethyl)]carbamate amylose	Chiralpak AS	D
Type IIIB		
Poly(*N*-1-acryloylphenylalanine ethylester)	Chiraspher	M
Poly(triphenylmethylmethacrylate)	Chiralpak OT(+)	D
Poly(2-pyridyl-diphenylmethylmethacrylate)	Chiralpak OP(+)	D
Type IV		
Bovine serum albumin	Resolvosil-BSA-7	MN
α_1-Glycoproteic acid	Enantiopac	LKB
	Chiral-AGP	CT
Human serum albumin	Chiral protein 2	SFCC
Ovomucoide	Ultron ES-OVM	MM
Vancomycin	Chirobiotic V	A
Teicoplanin (macrocyclic antibiotics)	Chirobiotic T	A
Cellobiohydrolase (stable enzyme)	Chiral CBH	A

Suppliers: A, Astec; B, Baker; CT, ChromTech AB; D, Daicel; JW, J&W Scientific; LKB; M, Merck; MM, MAC-MOD Analytical; MN, Macherey-Nagel; R, Regis; Sed, SEDERE; Ser, Serva; Sum, Sumitomo; Sup, Supelco.

solute-CSP complex involves inclusion of the solute in the chiral cavities acting cooperatively. Group III CSPs (Table 1) can be applied in SFC.

Group IV Group IV contains protein and antibiotic-grafted silica. As for the phases in sub-group Ib, these CSPs cannot be used in SFC.

Applications

The most interesting applications of SFC concern the type IA and III CSPs, and to a lesser extent type II CSPs.

Group IA

As a general rule, most applications concern the brush-type CSPs having a π-electron acceptor character. This is because many compounds of pharmaceutical interest contain a π-donor group.

CSPs derived from *N*-(3,5-dinitrobenzoyl)amino acids are among the most widely used for enantiomeric separations of numerous compounds. The early commercialization of the well-known (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine-derived CSP ((*R*)-DNBPG), designed by Pirkle and co-workers in 1980, and the easy and inexpensive preparation of this type of CSP, has prompted many researchers to design new π-acid CSPs. Although the scope of applications of these CSPs does not vary very much, all workers agree that small structural changes in the phases can have significant effects on the chromatographic behaviour. Our laboratories have been involved in the development of CSPs derived from tyrosine. Among them, a 'broad-spectrum' CSP has been marketed under the

registered name ChyRoSine-A and an improved version of this has been described. Their enantiomer-recognition abilities have been evaluated both by LC and SFC and the scope of applications including numerous racemates such as benzodiazepines, sulfoxides, phosphine oxides, lactams and β-blockers demonstrated.

An anthrylamine derivative adsorbed onto porous graphitic carbon has been used to separate two commercial anti-inflammatory agents (ibuprofen and flurbiprofen) and a series of racemic tropic acid derivatives. The enantioselective properties of this material were compared with the corresponding silica-based CSP and it was concluded that the former was more efficient.

π-Basic CSPs, deriving from tyrosine and bearing two stereogenic centres, were designed and successfully applied to the enantioseparation of pharmaceutical compounds using SFC. Warfarin and ICI 176334 (a potential nonsteroidal antiandrogen used in the treatment of prostate cancer) were baseline-resolved on these phases without any prior derivatization step into 3,5-dinitrobenzoyl derivatives. Several π-donor CSPs, with (*R*)-*N*-pivaloylnaphthylethylamide as the chiral selector group, have been applied to SFC.

Valine-diamide phases have been used in SFC for the enantioseparation of racemic *N*-4-nitrobenzoylamino acid isopropyl esters. The enantioselectivity in SFC was comparable to that in LC using the mixture 2-propanol/*n*-hexane as mobile phase but the time required for analysis was less than 5 min by SFC.

As a general rule, the use of SFC does not improve enantioselectivity for type I CSPs. The selectivities obtained in LC and SFC are identical, showing that the chiral recognition mechanisms are the same for hexane and carbon dioxide. In this case, the advantage of SFC over LC is of a kinetic nature, giving higher efficiency per unit time and therefore faster analysis. **Figure 2** illustrates the kinetic advantage of SFC over LC by showing the separation of the enantiomers of Oxazepam on ChyRoSine-A in both LC and SFC. At constant resolution, the analysis time by SFC is 6 min, and 24 min by LC. However, it must be emphasized that in some cases different selectivities between LC and SFC are encountered.

The first of these cases concerns the nonconventional separation of π-acceptor solutes on π-acceptor CSPs. In such a case, π–π charge transfer interactions cannot take place during the chiral

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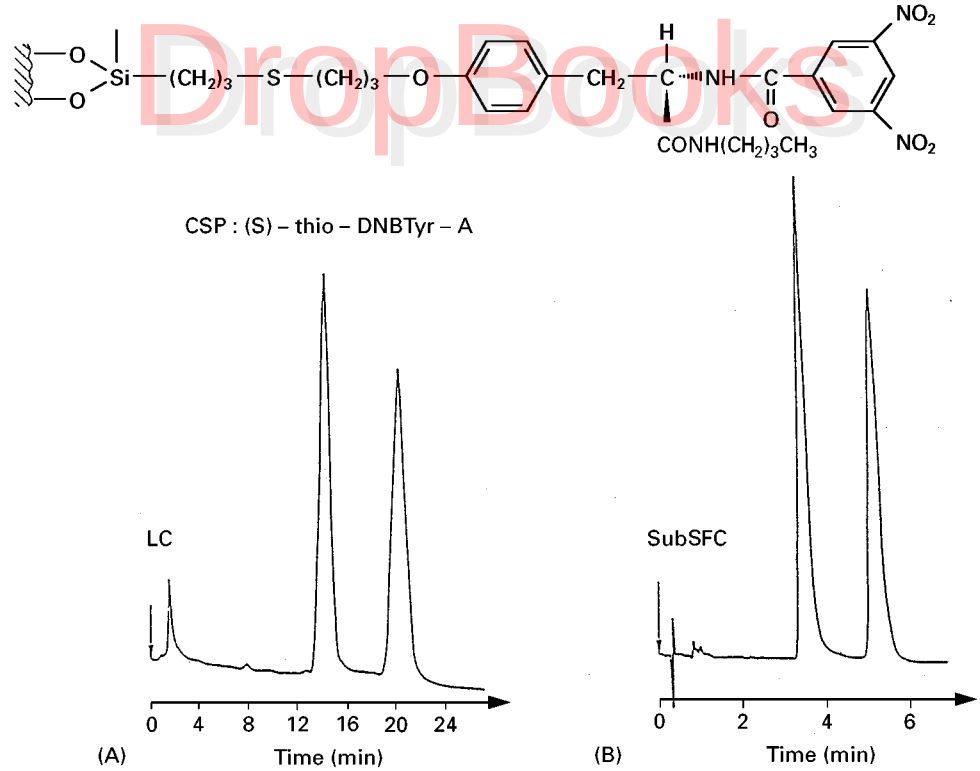

Figure 2 LC (A) and SFC (B) separations of the enantiomers of oxazepam using a ChyRoSine-A CSP: a comparison of analysis time at constant resolution ($R_s = 3.5$). Operating conditions: 150 × 4.6 mm i.d. column packed with 5 μm ChyRoSine-A CSP. LC: mobile phase, hexane/ethanol (90 : 10); flow rate, 2 mL min^{-1}. SubSFC: mobile phase, carbon dioxide/ethanol (92 : 8); flow rate, 4.5 mL min^{-1} at 0°C; outlet pressure 200 bar. Temperature, 25°C; UV detection at 229 nm. (Reproduced from Bargmann-Leyder N, Tambuté A and Caude M (1992) Chiralité et chromatographie en phase supercritique. A review. *Analysis* 20: 189–200.)

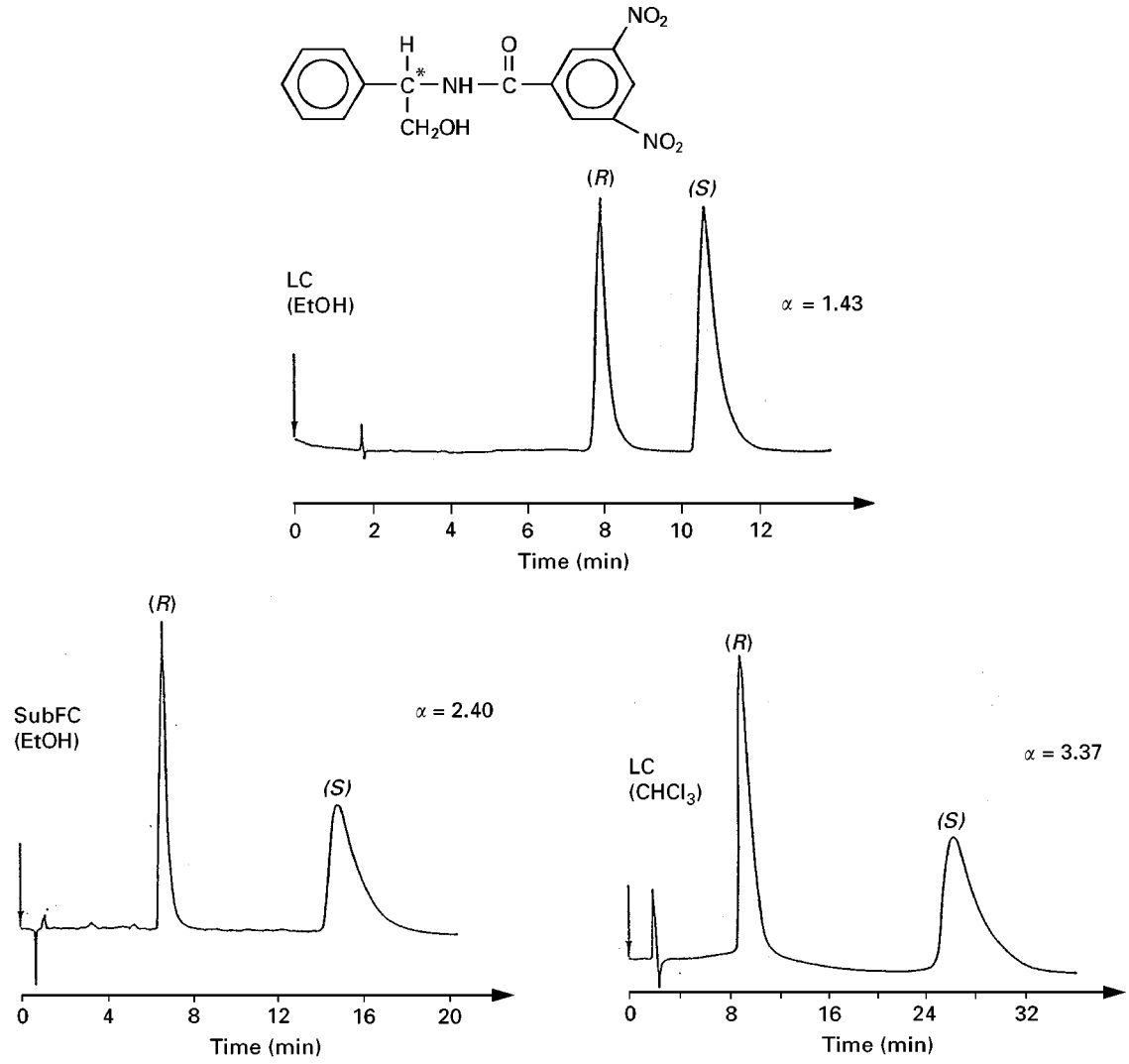

Figure 3 Influence of the nature of the mobile phase on the resolution of *N*-(3,5-dinitrobenzoyl)phenylglycinol on (*R*)-DNBPG. LC conditions: mobile phase, hexane/ethanol (85 : 15, v/v) ($k'(r) = 3.9$, $k'(s) = 5.6$) or hexane/chloroform (10 : 90, v/v) ($k'(r) = 4.3$, $k'(s) = 16.3$); flow rate 2 mL min^{-1}; temperature, 25°C; UV detection at 254 nm. SubSFC conditions: mobile phase, carbon dioxide/ethanol (93 : 7, w/w); flow rate 4.5 mL min^{-1} at 0°C; average column pressure, 200 bar; temperature, 25°C; UV detection at 254 nm. (Reproduced from Macaudière P, Lienne M, Caude M, Rosset R and Tambuté A (1989) Resolution of π-acid racemates on π-acid chiral stationary phases in normal-phase liquid and subcritical fluid chromatographic modes. A unique reversal of elution order on changing the nature of the achiral modifier. *Journal of Chromatography* 467:357–372, with permission from Elsevier Science.)

recognition mechanism. This is why the main mechanism may vary depending on the mobile phase composition, sometimes resulting in a reversal of the elution order. As shown in **Figure 3**, important discrepancies in the selectivity values are noted between hexane/ethanol in LC and the supercritical mobile phase carbon dioxide/ethanol. The chromatographic behaviour observed in SFC is somewhat similar to that observed in LC with hexane/methylene chloride/chloroform mobile phases.

The second major exception concerns the separation of β-blockers using ChyRoSine-A as CSP. Surprisingly, the direct separation of a series of β-blockers was achieved on commercially available ChyRoSine-A CSP and on its improved version, whereas these solutes appear to be unresolved or poorly resolved by normal-phase liquid chromatography (**Figure 4**; **Table 2**). The chromatographic behaviour (both in SFC and LC) of various propranolol analogues has been thoroughly studied and further spectroscopic investigations carried out. Starting from these data, detailed chiral recognition mechanisms have been proposed, based on molecular modelling. The solute conformations are selected by taking into account the information provided by the ^{1}H NMR spectra and it appears that the solvating

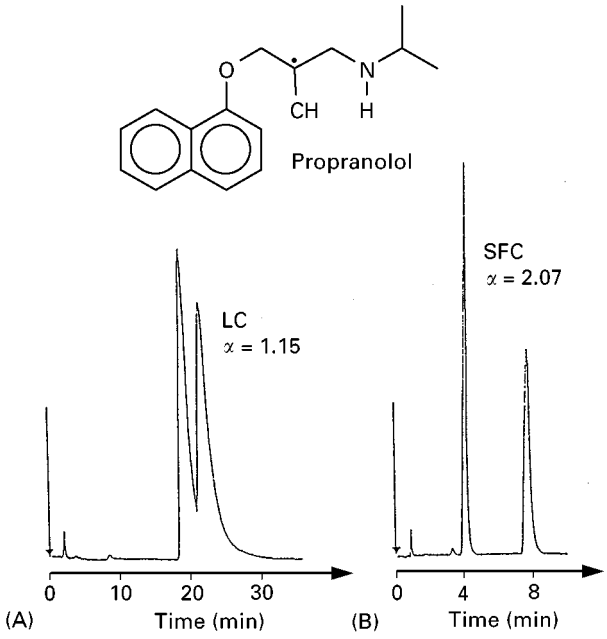

Figure 4 Comparative chromatograms of the resolution of propranolol on ChyRoSine-A CSP by LC (A) and SFC (B). Operating conditions: 150 × 4.6 mm i.d. column packed with 5 μm ChyRoSine-A CSP. LC. mobile phase/hexane/ethanol containing 1% v/v of *n*-propylamine (95 : 5, v/v); flow rate 1 mL min^{-1}. SFC: mobile phase, carbon dioxide/ethanol containing 1% v/v of *n*-propylamine (90 : 10); flow rate, 4 mL min^{-1} at 0°C, outlet pressure 200 bar. Room temperature; UV detection at 224 nm. (Reproduced with permission from Siret L, Bargmann N, Tambuté A and Caude M (1992) Direct enantiomeric separation of β-blockers on ChyRoSine-A by supercritical fluid chromatography: supercritical carbon dioxide as transient *in situ* derivatizing agent. *Chirality* 4: 252–262.)

effect of carbon dioxide induces a change in conformation of propranolol (**Figure 5**). This change occurs in the presence of carbon dioxide but only if the solute bears both an amino proton and an ether function separated by three carbon atoms. Without carbon dioxide, (*R*)- and (*S*)-propranolol conformers have geometrical structures such that the chiral recognition process is poor: the chiral centre of the solute cannot develop stereoselective interactions with the CSP and the interactions involved are the same for both enantiomers (**Figure 6**). On the other hand, the conformation of propranolol in the presence of carbon dioxide is geometrically favourable to the chiral discrimination. The conformations of the chiral stationary phase, (*R*)-solute, (*S*)-solute and their respective associations are shown in **Figure 7**. In this case, the (*R*)-propranolol conformer involves higher energy interactions with (*S*)-CSP than the (*S*) conformer.

High speed chiral separations (analysis duration < 1.5 min) of β-blockers have been achieved using a short packed column and a high mobile phase flow rate. The use of high speed chiral separations allows a decrease in solvent consumption (CO_2 and polar modifier), and by minimizing band broadening in the column gives better detectability. As an example, **Figure 8** shows the enantioseparation of propranolol and pindolol. These results again demonstrate the kinetic superiority of the SFC over LC. Moreover, in the case of β-blockers, the better kinetics of SFC is combined with enhanced thermodynamics owing to the favoured chiral recognition provided by the conformation of the molecules in the presence of carbon dioxide.

Finally, it should be noted that type I CSPs have been successfully applied to preparative SFC.

Group II

The scope of applications of a given cyclodextrin is determined by the goodness of the fit between the chiral cavity and the size of the solute to be resolved. If the cavity is too large compared with the solute, there is no preferred orientation (and therefore no selectivity); on the other hand, if the cavity is too small, there is no solute inclusion. Most separations are therefore achieved using β-cyclodextrin for which the internal diameter of the cavity (0.78 nm) is well suited to naphthyl, biphenyl, benzoyl or cyclohexyl moieties present in numerous molecules of pharmaceutical interest. γ-Cyclodextrin is well adapted to molecules bearing large substituents (such as phenobarbital); α-cyclodextrin is preferentially used for smaller molecules bearing a single aromatic group or a small aliphatic chain.

Cyclodextrin CSPs have been used for SFC, although they are, *a priori*, better adapted to the separation of enantiomers in reversed-phase LC. In fact, in normal-phase liquid chromatography, the hydrophobic solvent, e.g. hexane, chloroform, etc., occupies the cyclodextrin cavity and cannot easily be displaced by solutes. The average behaviour of the column is then somewhat similar to that of a diol column (almost no chiral resolution has been obtained using cyclodextrin phases in normal-phase liquid chromatography). The small size of the carbon dioxide molecule means that it can be displaced more easily than other apolar solvents such as hexane from the cyclodextrin cavities. Moreover, the carbon dioxide molecule exhibits an induced dipole moment, giving it a higher polarity than hexane.

The use of polar modifiers induces a decrease in retention (polar modifier competes with the solute and increases the solubility of the solute). In terms of selectivity, all the polar modifiers that have been used (methanol, ethanol, 1-butanol, 2-butanol,

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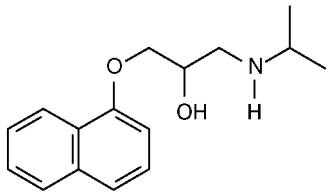
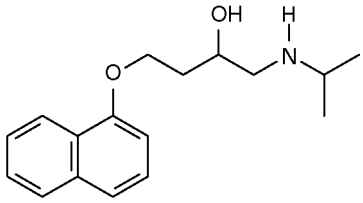
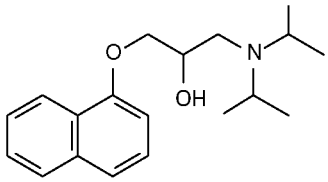
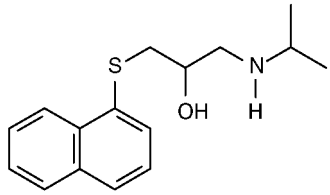
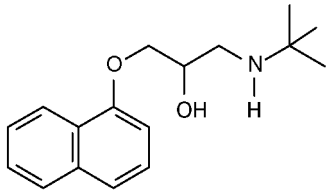
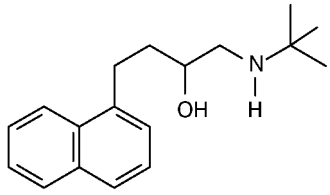
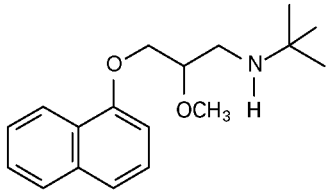
Table 2 Chromatographic data for the resolution of propranolol and some analogues on ChyRoSine-A CSP by LC and SFC

Compounds	*LC*			*SFC*		
	% polar modifier	k_2	α	*% polar modifier*	k_2	α
1 	5	11.7	1.14	12	19.8	2.07
2 	5	15.5	1	12	12.8	1.07
3 	5	13.2	1	12	11.3	1
4 	2.5	10.7	1	12	10.9	1.07
5 	5	9.72	1.32	12	24.7	2.27
6 	5	9.2	1	12	13.2	1.08
7 	5 2.5	1.7 2.3	1.01 1.03	12	13.9	1.47

Operating conditions: column 150 × 4.6 mm i.d., UV detection 224 nm. LC: mobile phase hexane/ethanol containing 1% (v/v) of *n*-propylamine, the percentage (v/v) of polar modifier in hexane is indicated in the table; room temperature; flow rate 2 mL min^{-1}. SFC: mobile phase carbon dioxide/methanol containing 1% (v/v) of *n*-propylamine, the percentage (v/v) of polar modifier in CO_2 is indicated in the table; temperature 25°C; average column pressure 180 bar; flow rate at 0°C 4 mL min^{-1}.

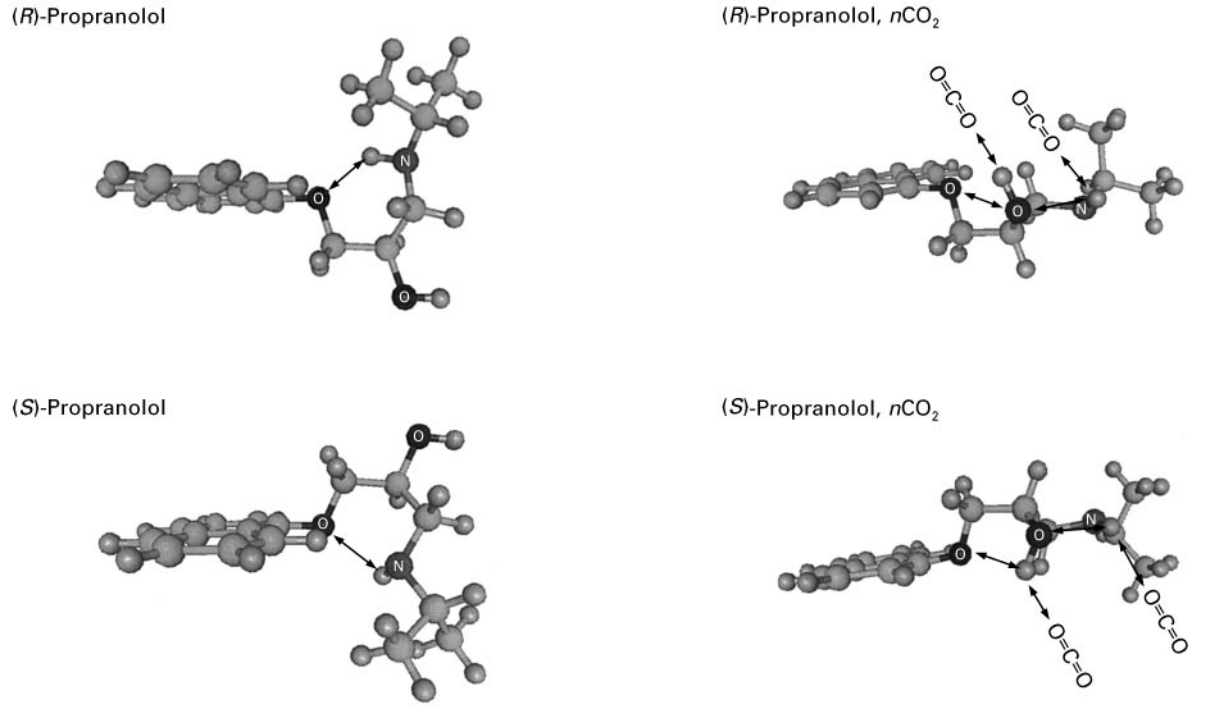


Figure 5 Change of the propranolol conformation induced by carbon dioxide. (A) Optimized structures of (*R*)- and (*S*)-propranolol without CO_2. The intramolecular hydrogen bonding is by an arrow. (B) Optimized structures of (*R*)- and (*S*)-propranolol with CO_2. In order to simplify the figure, only two molecules of carbon dioxide are illustrated. (Reproduced with permission from Bargmann-Leyder N, Sella C, Bauer D, Tambuté A and Caude M (1995) Separation of β-blockers using supercritical fluid chromatography: investigation of the chiral recognition mechanism using molecular modelling. *Analytical Chemistry* 67: 952–958.)

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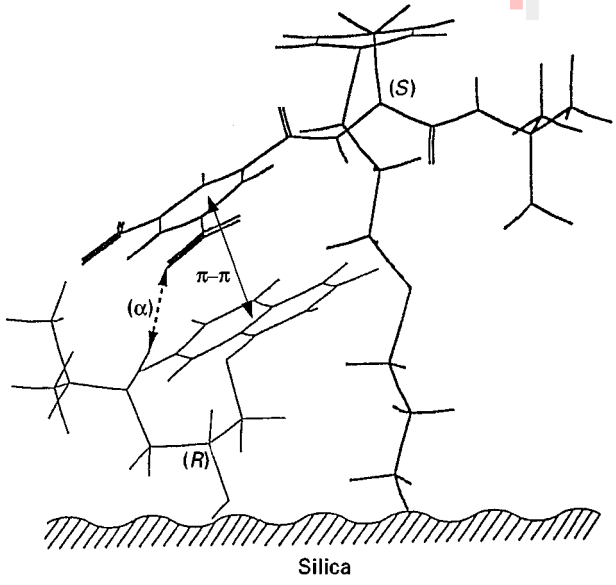

Figure 6 Optimized association between the optimized structures of (*R*)-propranolol (without carbon dioxide) and ChyRoSine-A CSP. (Reproduced with permission from Bargmann-Leyder N, Sella C, Bauer D, Tambuté, A and Caude M (1995) Separation of β-blockers using supercritical fluid chromatography: investigation of the chiral recognition mechanism using molecular modelling. *Analytical Chemistry* 67: 952–958.)

2-propanol) are almost equivalent. Water should not be used since it decreases selectivity significantly.

As reported by Macaudière and co-workers, the use of cyclodextrin CSPs in SFC allows particular selectivities to be obtained. A comparison between solute behaviour in reversed-phase and in normal-phase liquid chromatography clearly demonstrated that SFC and reversed-phase liquid chromatography are two complementary techniques; this result has widened the range of cyclodextrin phase applications. **Figure 9** shows the comparison of the separation of the 2-naphthyl and *o*-anisyl phosphine oxides on Cyclobond I in normal-phase LC and SFC. No or very weak enantiomeric resolution is achieved using normal-phase LC.

Enantiomeric separation of a variety of drugs and related compounds (ancymidol, coumachlor, ibuprofen, mephenytoin, tropicamide, verapamil, etc.) on an (*S*)-naphthylethylcarbamoylated-β-cyclodextrin phase using sub- and supercritical fluid chromatography has been accomplished by Williams and co-workers. Compounds previously resolved on native or derivatized cyclodextrin CSPs in LC using reversed-phase or polar organic mobile phases could be resolved in SFC using a simple carbon dioxide/methanol eluent. Resolution of cromakalim was

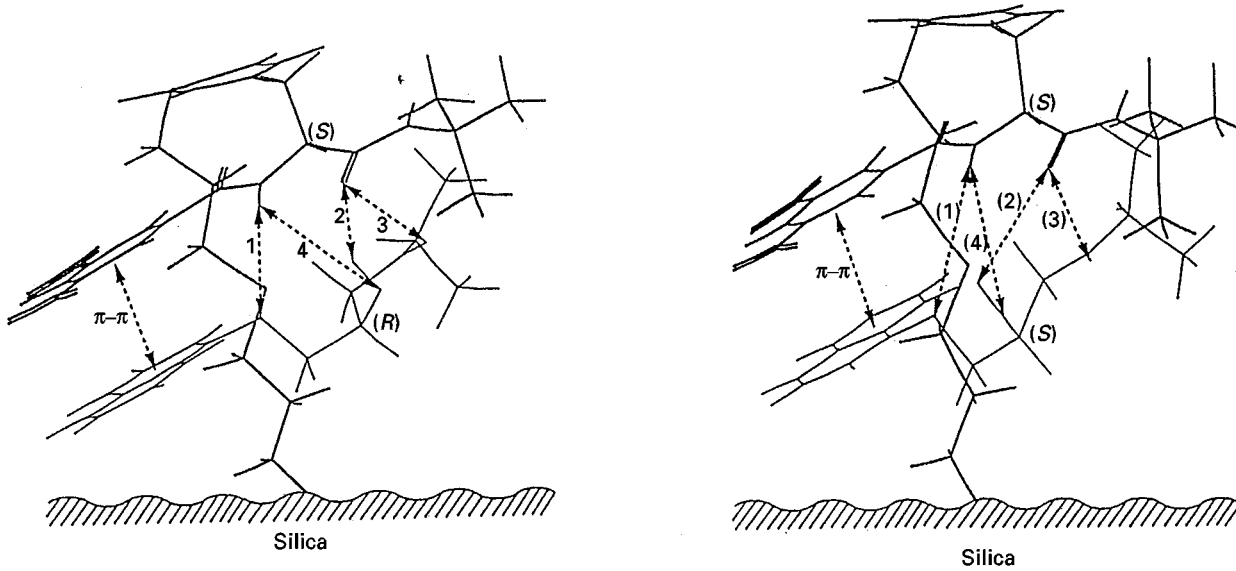

Figure 7 Optimized associations between the optimized structures of (*R*)- and (*S*)-propranolol (with carbon dioxide) and ChyRoSine-A CSP. (Reproduced with permission from Bargmann-Leyder N, Sella C, Bauer D, Tambuté A and Caude M (1995) Separation of β-blockers using supercritical fluid chromatography: investigation of the chiral recognition mechanism using molecular modelling. *Analytical Chemistry* 67: 952–958.)

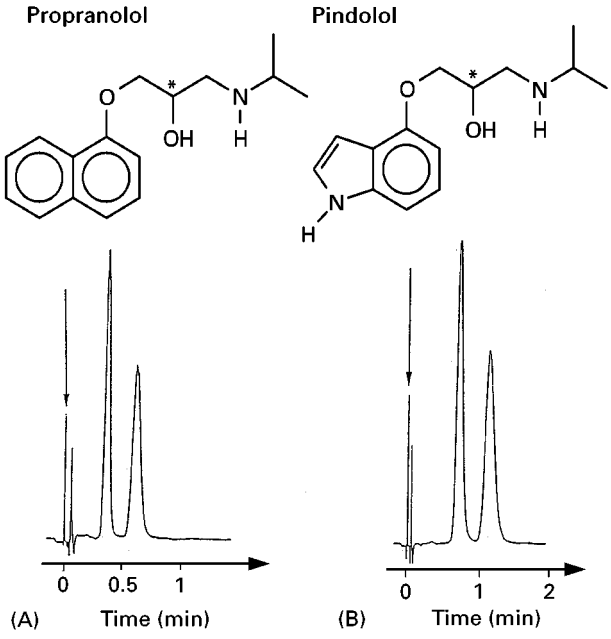

Figure 8 High-speed enantiomeric separation of (A) propranolol and (B) pindolol on ChyRoSine-A CSP. Operating conditions: column, 50 × 3.2 mm i.d.; mobile phase, carbon dioxide/(ethanol containing 1% (v/v) of *n*-propylamine) (80 : 20, (v/v); flow rate 7.5 mL min^{-1} at 0°C; temperature, 27°C; pressure, 220 bar; UV detection at 224 nm. (Reproduced with permission from Bargmann-Leyder N, Thiebaut D and Vergne F *et al.* (1995) High speed chiral separation of β-blockers by supercritical fluid chromatography on ChyRoSine-A. *Chromatographia* 39: 673–681.)

not obtained on the (*S*)-naphthylethylcarbamoylated-β-cyclodextrin CSP using LC, but was readily accomplished using SFC (**Figure 10**). The separation of the enantiomers of *N*-(3,5-dinitrobenzoyl)valine methyl ester, ancymidol and proglumide was also obtained in a single run using carbon dioxide/methanol eluent, whereas the same separations in LC required three different mobile phases.

Group III

Chiralcel-OD CSP tris(3,5-dimethylphenyl carbamate cellulose) has been used successfully for the SFC enantioseparation of β-blockers, potassium channel activator analogues and other compounds. A Chiralpak-AD column, tris(3,5-dimethylphenyl carbamate amylose), has been used to resolve enantiomeric mixtures of nonsteroidal anti-inflammatories.

Other CSPs derived from cellulose have been successfully applied to the SFC enantioseparation of compounds of pharmaceutical interest. For example, an intermediate in the synthesis of a drug targeted for cardiac arrhythmia was separated on Chiralcel-OB; the four optical isomers of a new calcium channel blocker, LF 2.0254, were resolved on Chiralcel-OJ; and some CSPs have been applied to the SFC separation of various frequently used drug racemates such as profens and barbiturate derivatives, benzodiazepines, etc.

A Chiralcel-OD-H column and an achiral aminopropyl column have been employed for the analysis of products formed in rat liver microsomal metabolism

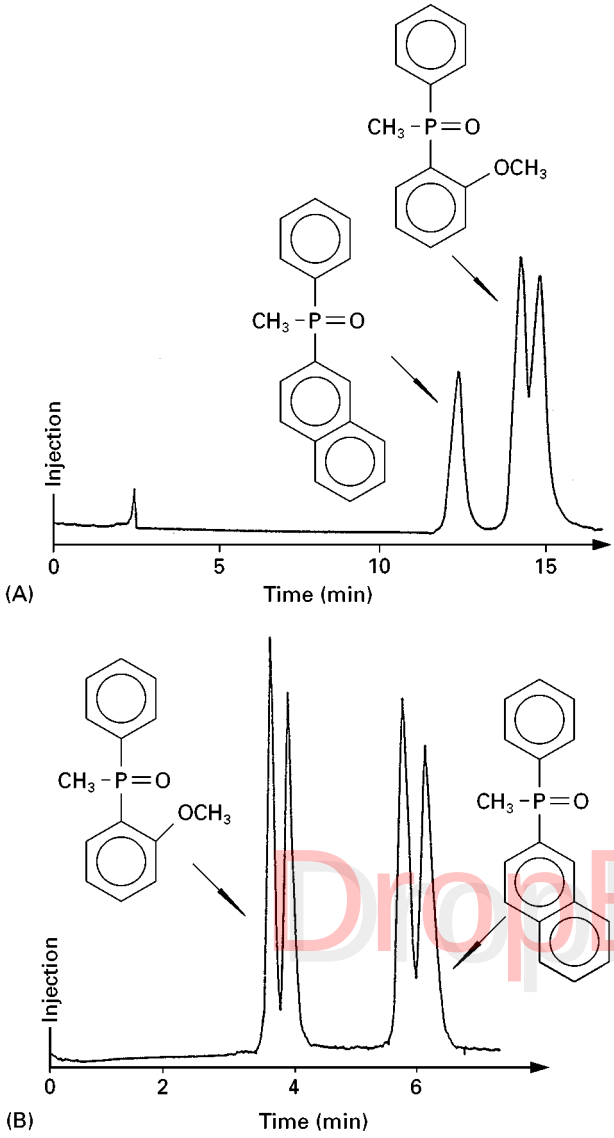

Figure 9 Comparison of the separation of the 2-naphthyl and *o*-anisyl phosphine oxides on Cyclobond I using LC and SFC. LC conditions: mobile phase, hexane/ethanol (85 : 15, v/v); flow rate, 1 mL min^{-1}; UV detection at 234 nm. SFC conditions: mobile phase, carbon dioxide/methanol (94 : 6, w/w); flow rate, 4.5 mL min^{-1}; temperature 25°C; pressure, 150 bar; UV detection at 234 nm. (Reproduced from Macaudière P, Caude M, Rosset R and Tambuté A (1987) Resolution of racemic amides and phosphine oxides on a β-cyclodextrin-bonded stationary phase by subcritical fluid chromatography. *Journal of Chromatography* 405:135–143, with permission from Elsevier Science.)

of racemic camazepam (a hypnotic/anxiolytic drug in clinical use) and the fast chiral separation of different compounds (oxprenolol, pindolol, warfarin) has been achieved by microbore SFC using a Chiralcel-OD type stationary phase.

Kot and co-workers proposed the serial coupling of different CSP columns (Chiralpak-AD, Chiralcel-OD and Chirex 3022 (brush-type with π-donor

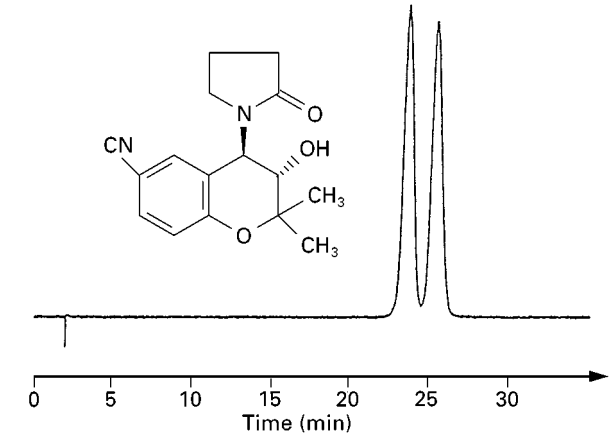

Figure 10 SFC separation of the cromakalim enantiomers on the (*S*)-naphthylethylcarbamoylated-β-cyclodextrin CSP. Operating conditions: mobile phase, carbon dioxide/methanol (96 : 4); flow rate, 2 mL min^{-1}; temperature 30°C; pressure, 15 MPa; UV detection at 254 nm. (Reproduced from Williams KL, Sander LC, and Wise SA (1996) Comparison of liquid and supercritical fluid chromatography using naphthylethylcarbamoylated-β-cyclodextrin chiral stationary phases. *Journal of Chromatography A* 746: 91–101, with permission from Elsevier Science.)

characteristics)). This coupling allowed the authors to achieve baseline separations with all solutes investigated, basic (β-blockers, benzodiazepines)

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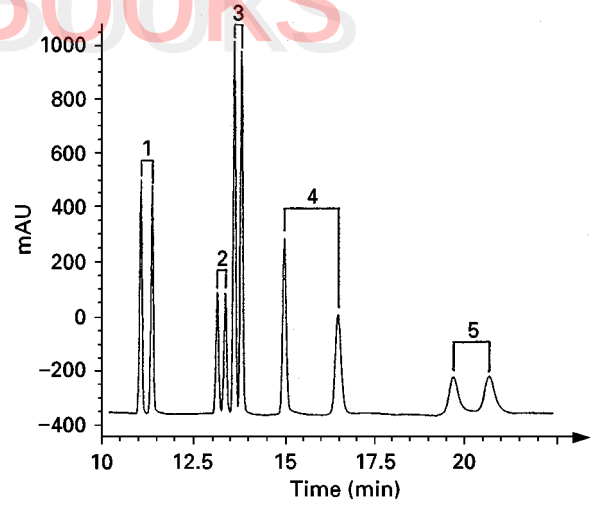

Figure 11 SFC separation of ibuprofen (1), fenoprofen (2), clenbuterol (3), propranolol (4) and lorazepam (5) using the serial coupling of different CSP columns. Operating conditions: columns, Chiralpak AD-Chiralcel OD-Chirex 3022; mobile phase, carbon dioxide/methanol (0.5% triethylamine + 0.5% trifluoroacetic acid) with methanol programmed from 4% (5 min) to 30% at 5% min^{-1}; flow rate 2 mL min^{-1}; temperature, 25°C; pressure, 200 bar. (Reproduced with permission from Kot A, Sandra P and Venema A (1994) Sub- and supercritical fluid chromatography on packed columns a versatile tool for the enantioselective separation of basic and acidic drugs. *Journal of Chromatographic Science* 32: 423–448.)

and acidic (nonsteroidal anti-inflammatory drugs, β-agonists). As an example, **Figure 11** shows the separation of ibuprofen, fenoprofen, clenbuterol, propranolol and lorazepam in a modifier-programmed run.

Systematic comparison of the chiral recognition mechanisms in LC and SFC for type III CSPs has been performed. It appears that, contrary to what occurs for type I CSPs, important discrepancies in selectivity values may exist between LC and SFC. The systematic comparison of LC and SFC for Chiralcel-OD and Chiralpak-AD CSPs demonstrates clearly that the presence of polar functional groups such as primary or secondary hydroxyl or amine functions may cause

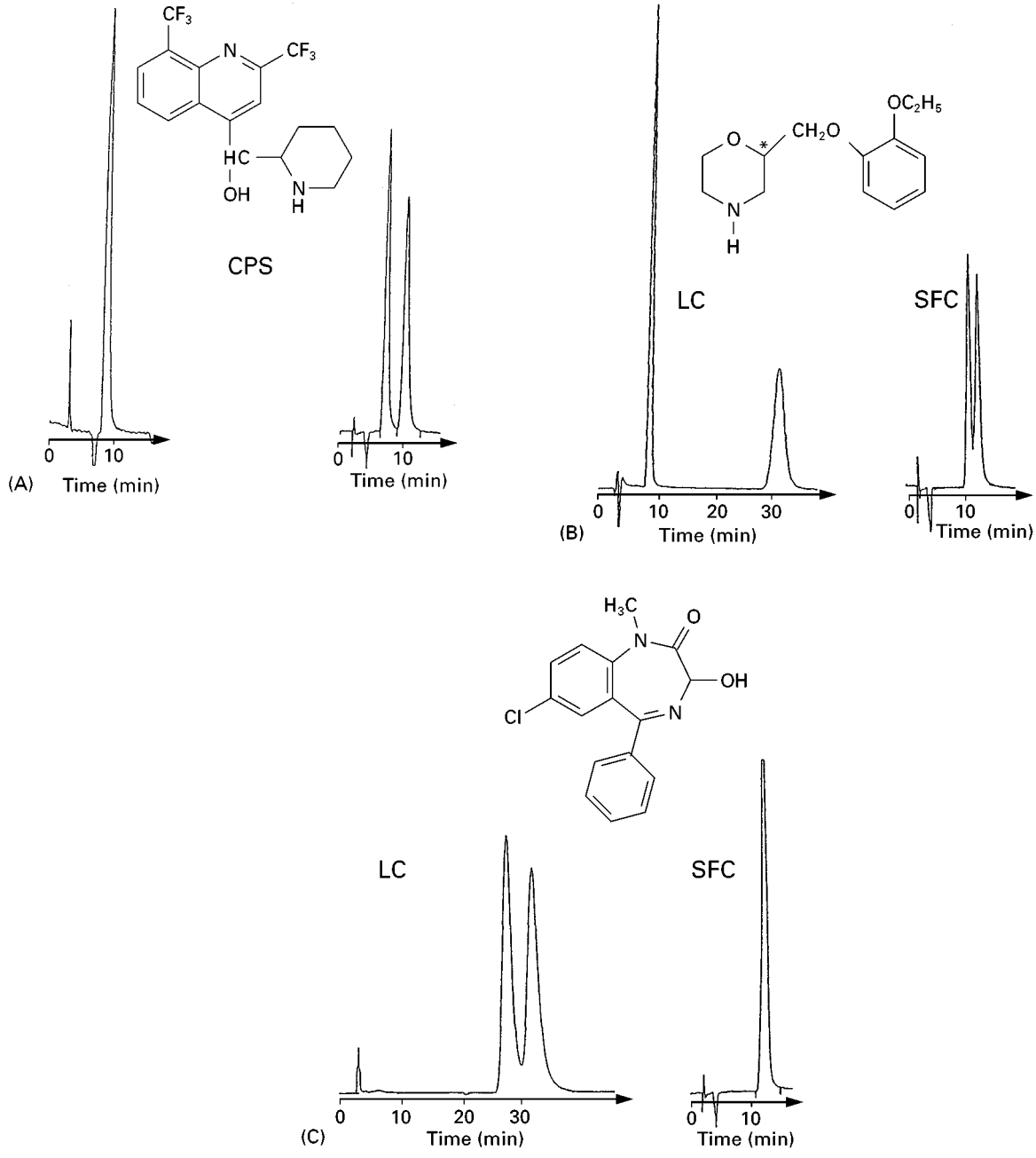

Figure 12 Comparison of LC and SFC for the separation of mefloquine (A), viloxazine (B) and temazepam (C) using Chiralcel OD CSP. Operating conditions: column, Chiralcel OD. LC, mobile phase hexane/ethanol containing 1% (v/v) of *n*-propylamine (90 : 10, v/v) for (A) and (C), 50 : 50 (v/v) for (B); flow rate 1 mL min^{-1}; room temperature. SFC: mobile phase, carbon dioxide/ethanol containing 1% (v/v) of *n*-propylamine 90 : 10 (v/v) for (A) and (B) 95 : 5 (v/v) for (C); flow rate, 2 mL min^{-1}; temperature, 25°C; pressure: 200 bar. UV detection. Separations are optimized for selectivity. (Reproduced with permission from Bargmann-Leyder N, Tambuté A and Caude M (1995) A comparison LC-SFC for cellulose and amylose-derived chiral stationary phases. *Chirality* 7: 311–325.)

large discrepancies in selectivity between LC and SFC. This result is peculiar to cellulose and amylose-derived CSPs, for which the interactions involved in chiral recognition are not always well balanced. Therefore, in the case of chiral resolution of polar solutes, the analyst should try both LC and SFC so that the more stereoselective one can be chosen. **Figure 12**A–C show some examples of the different selectivities that may exist between LC and SFC for polymer-type CSPs.

Other polymer-type CSPs have been used in SFC, such as those based on polymethacrylates of helical conformation and a polysiloxane CSP (polyWhelk-O), the 'polymeric version' of the commercially available brush-type CSP, Whelk-O 1. For the latter, the comparison was performed between the polymeric CSPs and its brush-type analogue, and it appeared that the polyWhelk-O CSP affords greater enantioselectivity and shorter retention under the same conditions.

Conclusion

Chiral separation is one of the fields where SFC is recognized to have better characteristics than HPLC, both from a kinetic and sometimes thermodynamic point of view.

In general, SFC offers faster separations than LC and often better selectivity values (particularly with cellulosic and amylosic polymer-type chiral stationary phases, and also with brush-type CSPs in particular cases). Consequently, SFC should be considered as a powerful analytical tool for the separation of basic and acidic drugs.

Capillary columns should be chosen for the analysis of chiral compounds having a low or medium polarity. On the other hand, packed columns are preferred for analytes of high polarity for which a polar modifier must be added to the supercritical carbon dioxide mobile phase.

Currently, to meet the requirements of quality control laboratories, most analyses are performed with packed columns. This is mainly due to the progress in SFC instrumentation (full control over many chromatographic parameters and particularly full control of the pressure). Analysts are looking for the chiral column that is best able to achieve racemate separation easily and in a single run. This objective will probably never be achieved, but we can expect that the serial coupling of chiral columns (two or three) will allow some progress in this direction owing to the kinetic advantage exhibited by SFC over LC.

Further Reading

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Synthetic Multiple Interaction ('Pirkle') Stationary Phases

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Introduction

Among the many types of chiral stationary phases (CSPs) that have been developed, synthetic multiple interaction (Pirkle-type, or brush-type) CSPs have proven to be among the most useful for many liquid chromatographic enantiomer separations. These CSPs consist of an enantioenriched small molecule selector immobilized on an inert chromatographic support, typically silica gel. Separation is achieved when the two enantiomers of the analyte are differentially adsorbed by the CSP (**Figure 1**). A combination